








Mobile colistin resistance *mcr-4.3*- and *mcr-4.6*-harbouring plasmids in livestock- and human-retrieved Enterobacterales in the Netherlands

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After the first report of the mobile colistin resistance gene *mcr-1* in 2015, 10 *mcr* variants were identified among antibiotic-resistant Enterobacterales and were mostly located on plasmids.^{1,2} The *mcr* genes encode phosphoethanolamine transferases capable of modifying Lipid A in membrane-associated LPS, thereby ultimately leading to colistin resistance.³ Colistin is considered a last-resort antibiotic for treatment of human Enterobacterales infections, but was also being used in veterinary medicine in the Netherlands in 2021.⁴ Until now, the presence of *mcr-4* has not been described before in The Netherlands. The major objective of this study was to analyse *mcr-4*-encoding plasmids from Enterobacterales obtained from humans and livestock in the Netherlands. To address this, a search for *mcr-4*-containing Enterobacterales isolates was performed in two surveillance databases containing Illumina short-read next-generation sequencing (NGS) data. These include the carbapenemase-producing Enterobacterales (CPE) surveillance collection from the National Institute for Public Health and the Environment (RIVM) and the livestock resistance monitoring collection from Wageningen Bioveterinary Research (WBVR). In addition, two clinical isolates of the Zuyderland Medical Center (ZMC) were analysed by the Maastricht University Medical Centre (MUMC).

The national CPE surveillance collection of the RIVM was searched for unique CPE and non-CPE isolates in the period from 2012 until 2020, in which only the first bacterial species and carbapenemase allele combination per person were included, yielding 3008 Enterobacterales isolates. This revealed two *Enterobacter* spp. (0.07%) with MLST ST54 that carried *mcr-4.3*. Average nucleotide identity (ANI) calculation using <http://enve-omics.ce.gatech.edu/ani/index> of the *Enterobacter* sp. ST54 with *Enterobacter kobei* strain UCI 23 (GenBank accession NZ_KI973153.1) revealed an ANI of 99.09%, demonstrating

that the *mcr-4.3*-carrying isolates were *E. kobei*. The collection of the WBVR in the period from 2018 until 2021 revealed that 4 out of 1200 veal calf isolates (0.3%) harboured *mcr-4.6*. Three *mcr-4.6*-containing plasmids, one from *Hafnia paralvei* and two from *Escherichia coli* were included in this study. The collection of the ZMC contained two *E. kobei* ST54 isolates with *mcr-4.3* isolated from hospitalized patients. Isolates were also subjected to Nanopore long-read sequencing by different in-house methods.^{5,6} Hybrid assembly of short-read and long-read sequencing data was performed using Unicycler (v.0.4.8, default settings) to reconstruct *mcr-4* plasmids, which were compared to internationally reported *mcr-4* plasmid sequences from NCBI (accessed 14 October 2022) using BioNumerics (v7.6.3).⁷ Hybrid *mcr-4* plasmid assemblies are available in GenBank (BioProject accession number PRJNA941496). Colistin resistance was confirmed using a standardised broth microdilution (BMD; Micronaut MIC strip colistin, Merlin), as previously described.⁸

Seven isolates carried *mcr-4*-like alleles and had varying resistomes (Figure 1a). In addition to *mcr-4.3*, the RIVM_C009363 isolate contained the *ant(2'')-Ia*, *bla_{ACT-9}*-like, *bla_{CTX-M-9}*, *fosA*, *mcr-9.1*, *qnrA1*, *sul1* and *tet(A)* resistance genes and lacked a carbapenemase gene. The RIVM_C014549 isolate was multi-resistant, containing the *aac(6')-Ib-cr*, *aac(6')-Ib3*, *aadA1*, *aph(3'')-Ib*, *aph(3'')-XV*, *aph(6)-Id*, *bla_{ACT-9}*, *bla_{SHV-12}*, *bla_{VIM-1}*, *catB2*, *dfrA14*, *fosA*, *mcr-9.1*, *mph(A)*, *qnrS1* and *sul1/2* genes. The *E. kobei* isolates MUMC-1 and MUMC-2 also belonged to MLST ST54, but were not clonally related to the RIVM_C014549 and RIVM_C009363 *E. kobei* isolates since they differed by 191 to 270 SNPs as inferred using split K-mer analysis using default settings. These isolates contained the *mcr-4.3*, *aph(6)-Id**, *aph(3'')-Ib**, *sul2**, *catA1**, *bla_{CTX-M-15*}*, *dfrA14*, *oqxA10*, *oqxB4*, *bla_{OXA-1}*, *aac(6')-Ib-cr*, *aac(3)-IIe*, *aadA1**, *mcr-9.1*, *qnrB1*, *bla_{ACT-64}*, *tet(A)* (*absent in

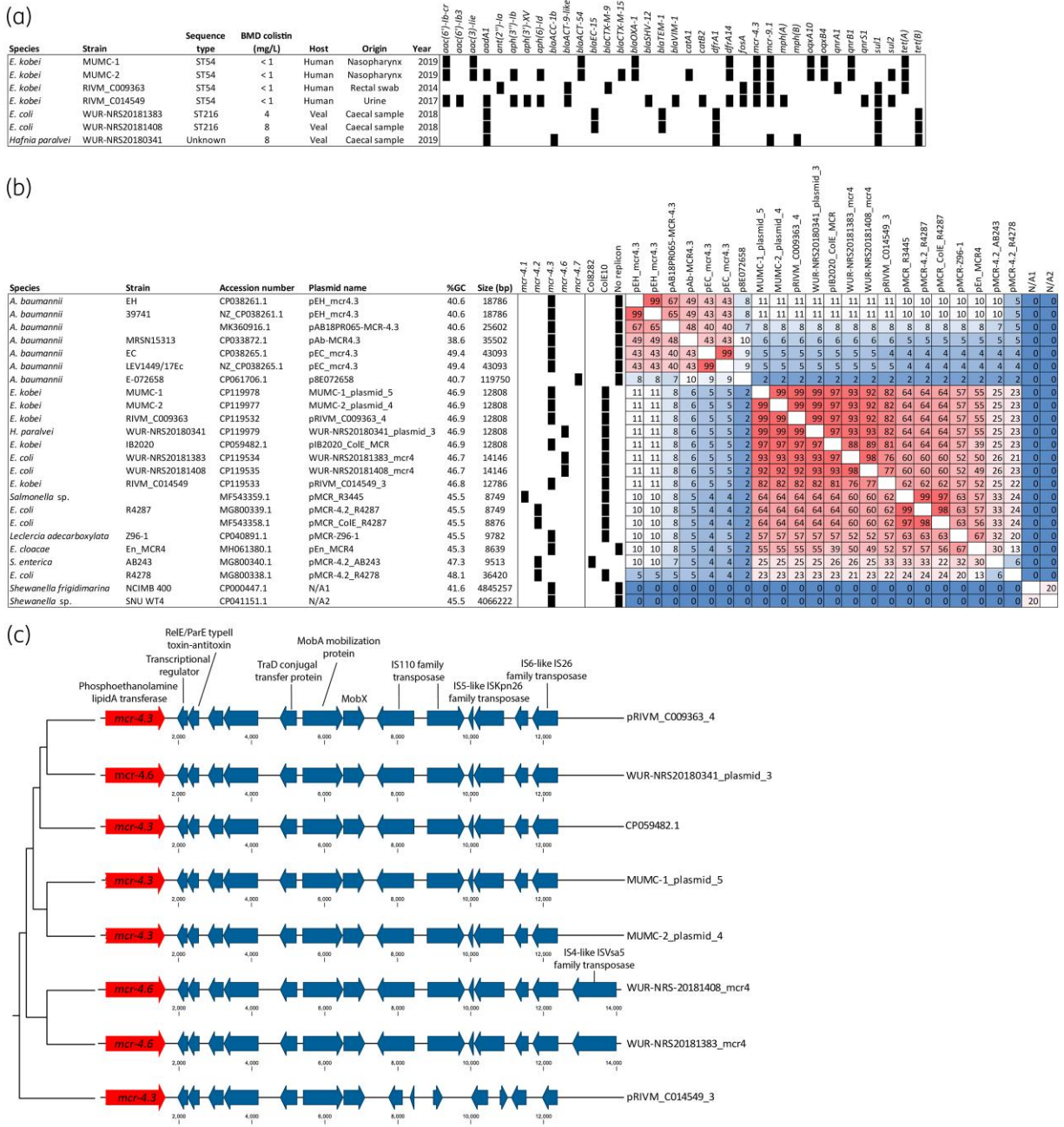


Figure 1. *In silico* characterization of *mcr-4*-containing plasmids. (a) Short-read sequencing-based molecular characteristics of the *mcr-4*-carrying isolates analysed in this study. (b) Comparison of *mcr-4* plasmids from the Netherlands in context with NCBI-retrieved plasmids. The % G+C content and plasmid size in bp is indicated. The presence of *mcr-4* alleles and replicons is indicated by squares. Heatmap indicating percentage identity. (c) Alignment of all *mcr-4*-harboring plasmids from the Netherlands with one *E. kobei* plasmid (pIB2020_ColE_MCR, CP059482.1) from Italy using CLC Genomics Workbench (v22.0.2). Plasmids were rotated and the *mcr-4* allele was set as starting point for comparison.

MUMC-1) resistance genes. The livestock *E. coli* isolates (WUR-NRS20181383 and WUR-NRS20181408) were from ST216, and contained the *aadA1*, *bla_{TEM-1}*, *bla_{EC-15}*, *dfrA1*, *sul1* and *tet(B)* resistance genes. The *H. paralvei* isolate (WUR-NRS20180341) contained the *aadA1*, *bla_{ACC-1b}*, *mph(B)*, *sul1*, *dfrA1*, *mcr-9.1* and *tet(B)* genes. None of the seven isolates carrying *mcr-4* contained

mutations in the *pmrAB* genes, known to be associated with colistin resistance, as inferred using PointFinder v4.1.⁹

Seven highly similar (76%–99%) and small (12.8–14.1 kb) *mcr-4* plasmids with a ColE10 replicon were identified with either an *mcr-4.3* allele in four human *E. kobei* isolates (MIC colistin ≤ 1 mg/L), or an *mcr-4.6* allele in three livestock-associated isolates

(MIC colistin 4–8 mg/L) collected between 2012 and 2021 (Figure 1b). More specifically, the MUMC-1_plasmid_5, MUMC-2_plasmid_4 and pRIVM_C009363_4 were 99% identical plasmids of 12.8 kb with a %G+C of 46.92%. The WUR-NRS20180341_plasmid_3, WUR-NRS20181383_mcr4 and WUR-NRS-20181408_mcr4 had slightly different %G+C and an *mcr-4.6* allele possibly conferring resistance to colistin. Four human *E. kobei* isolates (MUMC-01, MUMC-02, RIVM_C014549, RIVM_C009363) carried both *mcr-4.3* and *mcr-9*; *mcr-9* is not associated with colistin resistance in *Enterobacter*.^{10,11} Dutch livestock-retrieved isolates with *mcr-4.6* plasmids differed from the human-derived plasmids by one SNP in the *mcr-4.3* gene, leading to an *mcr-4.6* allelic variant, all exhibiting resistance to colistin.^{12,13} The *mcr-4* plasmids identified among Enterobacterales in the Netherlands were distinct from the *mcr-4* plasmids in the NCBI database (Figure 1b). However, one *E. kobei* plasmid (pIB2020_ColE_MCR, CP059482.1) from Italy from 2019 closely resembled the *mcr-4.3* and *mcr-4.6* plasmids from the Netherlands, suggesting international spread.¹⁴ The Dutch *mcr-4* plasmid architecture was comparable and comprised the phosphoethanolamine lipid A transferase gene *mcr-4*, followed by a type II toxin/antitoxin system, TraD conjugal transfer protein, MobA/X mobilization proteins and transposases (Figure 1c). The pRIVM_C014549_3 was different from the other *mcr-4* plasmids obtained in the Netherlands.

To test *mcr-4*-mediated colistin resistance, two strategies were employed.

Firstly, cloning of *mcr-4.3* and *mcr-4.6* with and without native promoter using primers MCR4_ownpromotorF: CACGGCAAAG ATCGGAGGG, MCR4_reverse: TCAGATCTCGTTGTAATTTCAAGG and MCR4_withoutownpromotorF: GAGGTCAGCTTGATTGTTTT into pGEM[®]-T Easy (Invitrogen) in *E. coli* DH10 β was performed according to the manufacturer's instructions. Plating was done on LB agar plates with 0, 0.125, 0.25, 0.5, 1 or 2 mg/L colistin and with ampicillin (50 mg/L). To avoid overexpression of the *mcr-4* genes, IPTG was omitted in the LB agar plates (no blue–white screening). No colonies were found after overnight incubation at 37°C on plates containing colistin. Also, induction with IPTG for the native and non-native *mcr-4* promoter constructs, did not result in colistin resistant colonies. Seven of the eight selected colonies that grew on LB agar plates with only ampicillin showed inverted *mcr-4* inserts, as determined by sequencing.

Secondly, transformation of an *Acinetobacter baumannii* (BioSample: ERS7182715, MIC colistin 0.5 mg/L) with *mcr-4.3* and *mcr-4.6* plasmid DNA isolated from isolate MUMC-1 and WUR_NRS20181383, respectively, was performed. After transformation of *A. baumannii* with either *mcr-4.3* or *mcr-4.6* plasmids, only colonies were observed on plates with colistin concentration up to and including 0.25 mg/L, indicating there was no change in colistin resistance for this strain.

In summary, the occurrence of *mcr-4* plasmids among Enterobacterales is low in the Netherlands. The *mcr-4.3* allele in *E. kobei* and the *mcr-4.6* allele in livestock *E. coli* and *H. parveii* likely do not encode for colistin resistance in the human and livestock isolates. A recent study also failed to detect colistin resistance in *E. kobei* ST54 co-harboring *mcr-4.3* and *mcr-9* and is in line with this study.¹⁵ In contrast, *mcr-4.3* conferred colistin resistance in *A. baumannii* and *Acinetobacter nosocomialis*,^{16,17}

suggesting species-specific functionality of this colistin resistance gene, but could not be confirmed in the *A. baumannii* isolate analysed in this study. The *mcr-4* plasmids from human and livestock were virtually identical, suggesting unnoticed horizontal dissemination of these plasmids in the Netherlands.

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Transparency declarations

The authors have nothing to disclose.

Ethics

Ethical approval was not needed for the study, since it was based on surveillance data only. Samples from which the isolates were cultured were all taken as part of routine healthcare.

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